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TITLE (OF THE INVENTION) (280 characters max)		
Chlamydia Antigens and Corresponding DNA Fragments and Uses Thereof		
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Respectfully submitted,

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**TITLE OF INVENTION**

**CHLAMYDIA ANTIGENS AND CORRESPONDING DNA FRAGMENTS AND USES  
THEREOF**

## FIELD OF INVENTION

5 The present invention relates to *Chlamydia* antigens and corresponding DNA molecules, which can be used in methods to prevent and treat *Chlamydia* infection in mammals, such as humans.

## **BACKGROUND OF THE INVENTION**

Chlamydiae are prokaryotes. They exhibit morphologic and structural similarities to gram-negative bacteria including a trilaminar outer membrane, which contains lipopolysaccharide and several membrane proteins that are structurally and functionally analogous to proteins found in *E. coli*. They are obligate intra-cellular parasites with a unique biphasic life cycle consisting of a metabolically inactive but infectious extracellular stage and a replicating but non-infectious intracellular stage. The replicative stage of the life-cycle takes place within a membrane-bound inclusion which sequesters the bacteria away from the cytoplasm of the infected host cell.

*C. pneumoniae* is a common human pathogen, originally described as the TWAR strain of *Chlamydia psittaci* but subsequently recognised to be a new species. *C. pneumoniae* is antigenically, genetically and morphologically distinct from other chlamydia species (*C. trachomatis*, *C. pecorum* and *C. psittaci*). It shows 10% or less DNA sequence homology with either of *C. trachomatis* or *C. psittaci* and so far appears to consist of only a single strain, TWAR.

*C. pneumoniae* is a common cause of community acquired pneumonia, only less frequent than *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* (Ref 1,2). It can also cause upper respiratory tract symptoms and disease, including bronchitis and sinusitis (Ref 1,3,4,5). The great majority of the adult population (over 60%) has antibodies to *C. pneumoniae* (Ref 5), indicating past infection which was unrecognized or asymptomatic.

Of considerable importance is the association of atherosclerosis and *C. pneumoniae* infection. There are several epidemiological studies showing a correlation of previous infections with *C. pneumoniae* and heart attacks, coronary artery and carotid artery disease (Ref 6-10). Moreover, the organisms has been detected in atherosomas and fatty streaks of the

coronary, carotid, peripheral arteries and aorta (Ref 11-15). Viable *C. pneumoniae* has been recovered from the coronary and carotid artery (Ref 16,17). Furthermore, it has been shown that *C. pneumoniae* can induce changes of atherosclerosis in a rabbit model (Ref 18). Taken together, these results indicate that it is highly probable that *C. pneumoniae* can cause atherosclerosis in humans, though the epidemiological importance of chlamydial atherosclerosis remains to be demonstrated.

5 A number of recent studies have also indicated an association between *C. pneumoniae* infection and asthma. Infection has been linked to wheezing, asthmatic bronchitis, adult-onset asthma and acute exacerbations of asthma in adults, and small-scale studies have shown that 10 prolonged antibiotic treatment was effective at greatly reducing the severity of the disease in some individuals (Ref 19-24).

In light of these results a protective vaccine against *C. pneumoniae* infection would be of considerable importance. There is not yet an effective vaccine for any human chlamydial infection. Nevertheless, studies with *C. trachomatis* and *C. psittaci* indicate that this is an attainable goal. For example, mice which have recovered from a lung infection with *C. trachomatis* are protected from infertility induced by a subsequent vaginal challenge (Ref 25). Similarly, sheep immunized with inactivated *C. psittaci* were protected from subsequent chlamydial-induced abortions and stillbirths (Ref 26). Protection from chlamydial infections has been associated with Th1 immune responses, particularly the induction of INF $\gamma$  producing CD4+ T-cells (Ref 27). The adoptive transfer of CD4+ cell lines or clones to nude or SCID mice conferred protection from challenge or cleared chronic disease (Ref 28,29), and in vivo depletion of CD4+ T cells exacerbated disease post-challenge (Ref 30,31). However, the presence of sufficiently high titres of neutralising antibody at mucosal surfaces can also exert a protective effect (Ref 32).

25 The extent of antigenic variation within the species *C. pneumoniae* is not well characterised. Serovars of *C. trachomatis* are defined on the basis of antigenic variation in MOMP, but published *C. pneumoniae* MOMP gene sequences show no variation between several diverse isolates of the organism (Ref 33-35). Regions of the protein known to be conserved in other chlamydial MOPMs are conserved in *C. pneumoniae* (Ref 33,34). One 30 study has described a strain of *C. pneumoniae* with a MOMP of greater than usual molecular weight, but the gene for this has not been sequenced (Ref 1). Partial sequences of outer

membrane protein 2 from nine diverse isolates were also found to be invariant (Ref 16). The genes for HSP60 and HSP70 show little variation from other chlamydial species, as would be expected. The gene encoding a 76kDa antigen has been cloned from a single strain of *C. pneumoniae*. It has no significant similarity with other known chlamydial genes (Ref 4).

5 Many antigens recognised by immune sera to *C. pneumoniae* are conserved across all chlamydiae, but 98kDa, 76 kDa and 54 kDa proteins may be *C. pneumoniae*-specific (Ref 2, 4, 36). Immunoblotting of isolates with sera from patients does show variation of blotting patterns between isolates, indicating that serotypes *C. pneumoniae* may exist (Ref 1,16). However, the results are potentially confounded by the infection status of the patients, since 10 immunoblot profiles of a patient's sera change with time post-infection. An assessment of the number and relative frequency of any serotypes, and the defining antigens, is not yet possible.

*C. pneumoniae* infection usually presents as an acute respiratory disease (i.e., cough, sore throat, hoarseness, and fever; abnormal chest sounds on auscultation). For most patients, the cough persists for 2 to 6 weeks, and recovery is slow. In approximately 10% of these cases, upper respiratory tract infection is followed by bronchitis or pneumonia. Furthermore, during a *C. pneumoniae* epidemic, subsequent co-infection with pneumococcus has been noted in about half of these pneumonia patients, particularly in the infirm and the elderly. As noted above, there is more and more evidence that *C. pneumoniae* infection is also linked to diseases other than respiratory infections.

20 The reservoir for the organism is presumably people. In contrast to *C. psittaci* infections, there is no known bird or animal reservoir. Transmission has not been clearly defined. It may result from direct contact with secretions, from fomites, or from airborne spread. There is a long incubation period, which may last for many months. Based on analysis of epidemics, *C. pneumoniae* appears to spread slowly through a population (case-to-case interval averaging 30 days) because infected persons are inefficient transmitters of the 25 organism. Susceptibility to *C. pneumoniae* is universal. Reinfections occur during adulthood, following the primary infection as a child. *C. pneumoniae* appears to be an endemic disease throughout the world, noteworthy for superimposed intervals of increased incidence (epidemics) that persist for 2 to 3 years. *C. trachomatis* infection does not confer cross-30 immunity to *C. pneumoniae*. Infections are easily treated with oral antibiotics, tetracycline or

erythromycin (2 g/d, for at least 10 to 14 d). A recently developed drug, azithromycin, is highly effective as a single-dose therapy against chlamydial infections.

In most instances, *C. pneumoniae* infection is often mild and without complications, and up to 90% of infections are subacute or unrecognized. Among children in industrialized countries, infections have been thought to be rare up to the age of 5 y, although a recent study (E Normann et al, Chlamydia pneumoniae in children with acute respiratory tract infections, *Acta Paediatrica*, 1998; Vol 87, Iss 1, pp 23-27) has reported that many children in this age group show PCR evidence of infection despite being seronegative, and estimates a prevalence of 17-19% in 2-4 y olds. In developing countries, the seroprevalence of *C. pneumoniae* antibodies among young children is elevated, and there are suspicions that *C. pneumoniae* may be an important cause of acute lower respiratory tract disease and mortality for infants and children in tropical regions of the world.

From seroprevalence studies and studies of local epidemics, the initial *C. pneumoniae* infection usually happens between the ages of 5 and 20 y. In the USA, for example, there are estimated to be 30,000 cases of childhood pneumonia each year caused by *C. pneumoniae*. Infections may cluster among groups of children or young adults (e.g., school pupils or military conscripts).

*C. pneumoniae* causes 10 to 25% of community-acquired lower respiratory tract infections (as reported from Sweden, Italy, Finland, and the USA). During an epidemic, *C. pneumoniae* infection may account for 50 to 60% of the cases of pneumonia. During these periods, also, more episodes of mixed infections with *S. pneumoniae* have been reported.

Reinfection during adulthood is common; the clinical presentation tends to be milder. Based on population seroprevalence studies, there tends to be increased exposure with age, which is particularly evident among men. Some investigators have speculated that a persistent, asymptomatic *C. pneumoniae* infection state is common.

In adults of middle age or older, *C. pneumoniae* infection may progress to chronic bronchitis and sinusitis. A study in the USA revealed that the incidence of pneumonia caused by *C. pneumoniae* in persons younger than 60 years is 1 case per 1,000 persons per year; but in the elderly, the disease incidence rose three-fold. *C. pneumoniae* infection rarely leads to hospitalization, except in patients with an underlying illness.

**SUMMARY OF THE INVENTION**

The present invention provides purified and isolated DNA molecules that encode *Chlamydia* polypeptides designated CPN100557 (SEQ ID No: 1,2), which can be used in methods to prevent, treat, and diagnose *Chlamydia* infection. The encoded polypeptides 5 include polypeptides having the amino acid sequence shown in SEQ ID No:3 and 4. Those skilled in the art will appreciate that the invention also includes DNA molecules that encode mutants and derivatives of such polypeptides, which result from the addition, deletion, or substitution of non-essential amino acids as described herein. The invention also includes RNA molecules corresponding to the DNA molecules of the invention.

10 In addition to the DNA and RNA molecules, the invention includes the corresponding polypeptides and monospecific antibodies that specifically bind to such polypeptides.

15 The present invention has wide application and includes expression cassettes, vectors, and cells transformed or transfected with the polynucleotides of the invention. Accordingly, the present invention provides (i) a method for producing a polypeptide of the invention in a recombinant host system and related expression cassettes, vectors, and transformed or transfected cells; (ii) a live vaccine vector, such as a pox virus, *Salmonella typhimurium*, or *Vibrio cholerae* vector, containing a polynucleotide of the invention, such 20 vaccine vectors being useful for, e.g., preventing and treating *Chlamydia* infection, in combination with a diluent or carrier, and related pharmaceutical compositions and associated therapeutic and/or prophylactic methods; (iii) a therapeutic and/or prophylactic method involving administration of an RNA or DNA molecule of the invention, either in a naked form or formulated with a delivery vehicle, a polypeptide or combination of polypeptides, or a monospecific antibody of the invention, and related pharmaceutical compositions; (iv) a 25 method for diagnosing the presence of *Chlamydia* in a biological sample, which can involve the use of a DNA or RNA molecule, a monospecific antibody, or a polypeptide of the invention; and (v) a method for purifying a polypeptide of the invention by antibody-based affinity chromatography.

**BRIEF DESCRIPTION OF THE DRAWINGS**

30 The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 shows the nucleotide sequence of the CPN100557 (SEQ ID No: 1 - entire sequence and SEQ ID No: 2 - coding sequence) and the deduced amino acid sequence of the CPN100557 protein from *Chlamydia pneumoniae* (SEQ ID No: 3 - full length and 4 - processed).

5 Figure 2 shows the restriction enzyme analysis of the gene encoding the *C. pneumoniae* CPN100557 gene.

#### DETAILED DESCRIPTION OF INVENTION

In the *C. pneumoniae* genome, open reading frames (ORFs) encoding chlamydial polypeptides have been identified. These polypeptides include polypeptides permanently found in the bacterial membrane structure, polypeptides that are present in the external vicinity of the bacterial membrane, include polypeptides permanently found in the inclusion membrane structure, polypeptides that are present in the external vicinity of the inclusion membrane, and polypeptides that are released into the cytoplasm of the infected cell. These polypeptides can be used in vaccination methods for preventing and treating *Chlamydia* infection.

According to a first aspect of the invention, there are provided isolated polynucleotides encoding the precursor and mature forms of *Chlamydia* polypeptides.

An isolated polynucleotide of the invention encodes (i) a polypeptide having an amino acid sequence that is homologous to a *Chlamydia* amino acid, the *Chlamydia* amino acid sequence being selected from the group consisting of:

20 (a) the amino acid sequences as shown: (SEQ ID No: 3 and 4).

The term "isolated polynucleotide" is defined as a polynucleotide removed from the environment in which it naturally occurs. For example, a naturally occurring DNA molecule present in the genome of a living bacteria or as part of a gene bank is not isolated, but the same molecule separated from the remaining part of the bacterial genome, as a result of, e.g., 25 a cloning event (amplification), is isolated. Typically, an isolated DNA molecule is free from DNA regions (e.g., coding regions) with which it is immediately contiguous at the 5' or 3' end, in the naturally occurring genome. Such isolated polynucleotides could be part of a vector or a composition and still be isolated in that such a vector or composition is not part of its natural environment.

30 A polynucleotide of the invention can be in the form of RNA or DNA (e.g., cDNA, genomic DNA, or synthetic DNA), or modifications or combinations thereof. The

DNA can be double-stranded or single-stranded, and, if single-stranded, can be the coding strand or the non-coding (anti-sense) strand. The sequence that encodes a polypeptide of the invention as shown in SEQ ID NOs: 1 and 2, can be (a) the coding sequence as shown in SEQ ID NOs:2 (b) a ribonucleotide sequence derived by transcription of (a) ; or (c) a different 5 coding sequence; this latter, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptides as the DNA molecules of which the nucleotide sequences are illustrated in SEQ ID NOs:1 to 2.

By "polypeptide" or "protein" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). Both terms 10 are used interchangeably in the present application.

By "homologous amino acid sequence" is meant an amino acid sequence that differs from an amino acid sequence shown in SEQ ID No: 3 or 4, only by one or more conservative amino acid substitutions, or by one or more non-conservative amino acid substitutions, deletions, or additions located at positions at which they do not destroy the specific antigenicity of the polypeptide.

Preferably, such a sequence is at least 75%, more preferably 80%, and most preferably 90% identical to an amino acid sequence shown in SEQ ID No: 3 or 4.

~~Homologous amino acid sequences include sequences that are identical or substantially identical to an amino acid sequence as shown in SEQ ID No:3 or 4. By "amino acid sequence substantially identical" is meant a sequence that is at least 90%, preferably 95%, more preferably 97%, and most preferably 99% identical to an amino acid sequence of reference and that preferably differs from the sequence of reference, if at all, by a majority of conservative amino acid substitutions.~~

Conservative amino acid substitutions typically include substitutions among amino 25 acids of the same class. These classes include, for example, amino acids having uncharged polar side chains, such as asparagine, glutamine, serine, threonine, and tyrosine; amino acids having basic side chains, such as lysine, arginine, and histidine; amino acids having acidic side chains, such as aspartic acid and glutamic acid; and amino acids having nonpolar side chains, such as glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, 30 tryptophan, and cysteine.

Homology is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Similar amino acid sequences are aligned to obtain the maximum degree of homology (i.e., identity). To this end, 5 it may be necessary to artificially introduce gaps into the sequence. Once the optimal alignment has been set up, the degree of homology (i.e., identity) is established by recording all of the positions in which the amino acids of both sequences are identical, relative to the total number of positions.

Homologous polynucleotide sequences are defined in a similar way. Preferably, a 10 homologous sequence is one that is at least 45%, more preferably 60%, and most preferably 85% identical to (i) a coding sequence of SEQ ID NOS:1 and 2.

Polypeptides having a sequence homologous to one of the sequences shown in SEQ ID NO: 3 or 4, include naturally-occurring allelic variants, as well as mutants or any other non-naturally occurring variants that are analogous in terms of antigenicity, to a polypeptide having a sequence as shown in SEQ ID NO: 3 or 4.

As is known in the art, an allelic variant is an alternate form of a polypeptide that is characterized as having a substitution, deletion, or addition of one or more amino acids that does not alter the biological function of the polypeptide. By "biological function" is meant the function of the polypeptide in the cells in which it naturally occurs, even if the function is not necessary for the growth or survival of the cells. For example, the biological function of a porin is to allow the entry into cells of compounds present in the extracellular medium. The biological function is distinct from the antigenic function. A polypeptide can have more than one biological function.

Allelic variants are very common in nature. For example, a bacterial species, 25 e.g., *C. pneumoniae*, is usually represented by a variety of strains that differ from each other by minor allelic variations. Indeed, a polypeptide that fulfills the same biological function in different strains can have an amino acid sequence that is not identical in each of the strains. Such an allelic variation may be equally reflected at the polynucleotide level.

Support for the use of allelic variants of polypeptide antigens comes from, e.g., 30 studies of the *Chlamydial* MOMP antigen. The amino acid sequence of the MOMP varies

from strain to strain, yet cross-strain antibody binding plus neutralization of infectivity occurs, indicating that the MOMP, when used as an immunogen, is tolerant of amino acid variations.

5 Polynucleotides, *e.g.*, DNA molecules, encoding allelic variants can easily be retrieved by polymerase chain reaction (PCR) amplification of genomic bacterial DNA extracted by conventional methods. This involves the use of synthetic oligonucleotide primers matching upstream and downstream of the 5' and 3' ends of the encoding domain. Suitable primers can be designed according to the nucleotide sequence information provided in SEQ ID NOs:1 and 2. Typically, a primer can consist of 10 to 40, preferably 15 to 25 nucleotides. It may be also advantageous to select primers containing C and G nucleotides in a proportion 10 sufficient to ensure efficient hybridization; *e.g.*, an amount of C and G nucleotides of at least 40%, preferably 50% of the total nucleotide amount.

Useful homologs that do not naturally occur can be designed using known methods for identifying regions of an antigen that are likely to be tolerant of amino acid sequence changes and/or deletions. For example, sequences of the antigen from different species can be compared to identify conserved sequences.

Polypeptide derivatives that are encoded by polynucleotides of the invention include, *e.g.*, fragments, polypeptides having large internal deletions derived from full-length polypeptides, and fusion proteins.

20 Polypeptide fragments of the invention can be derived from a polypeptide having a sequence homologous to any of the sequences shown in SEQ ID NO: 3 or 4, to the extent that the fragments retain the substantial antigenicity of the parent polypeptide (specific antigenicity). Polypeptide derivatives can also be constructed by large internal deletions that remove a substantial part of the parent polypeptide, while retaining specific antigenicity. 25 Generally, polypeptide derivatives should be about at least 12 amino acids in length to maintain antigenicity. Advantageously, they can be at least 20 amino acids, preferably at least 50 amino acids, more preferably at least 75 amino acids, and most preferably at least 100 amino acids in length.

30 Useful polypeptide derivatives, *e.g.*, polypeptide fragments, can be designed using computer-assisted analysis of amino acid sequences in order to identify sites in protein antigens having potential as surface-exposed, antigenic regions (Ref 37).

Polypeptide fragments and polypeptides having large internal deletions can be used for revealing epitopes that are otherwise masked in the parent polypeptide and that may be of importance for inducing a protective T cell-dependent immune response. Deletions can also remove immunodominant regions of high variability among strains.

It is an accepted practice in the field of immunology to use fragments and variants of protein immunogens as vaccines, as all that is required to induce an immune response to a protein is a small (e.g., 8 to 10 amino acid) immunogenic region of the protein. This has been done for a number of vaccines against pathogens other than *Chlamydia*. For example, short synthetic peptides corresponding to surface-exposed antigens of pathogens such as murine mammary tumor virus, peptide containing 11 amino acids; (Ref 38), Semliki Forest virus, peptide containing 16 amino acids (Ref 39), and canine parvovirus, 2 overlapping peptides, each containing 15 amino acids (Ref 40), have been shown to be effective vaccine antigens against their respective pathogens.

Polynucleotides encoding polypeptide fragments and polypeptides having large internal deletions can be constructed using standard methods (Ref 41), for example, by PCR, including inverse PCR, by restriction enzyme treatment of the cloned DNA molecules, or by the method of Kunkel *et al.* (Ref 42) biological material available at Stratagene.

A polypeptide derivative can also be produced as a fusion polypeptide that contains a polypeptide or a polypeptide derivative of the invention fused, e.g., at the N- or C-terminal end, to any other polypeptide (hereinafter referred to as a peptide tail). Such a product can be easily obtained by translation of a genetic fusion, i.e., a hybrid gene. Vectors for expressing fusion polypeptides are commercially available, such as the pMal-c2 or pMal-p2 systems of New England Biolabs, in which the peptide tail is a maltose binding protein, the glutathione-S-transferase system of Pharmacia, or the His-Tag system available from Novagen. These and other expression systems provide convenient means for further purification of polypeptides and derivatives of the invention.

Another particular example of fusion polypeptides included in invention includes a polypeptide or polypeptide derivative of the invention fused to a polypeptide having adjuvant activity, such as, e.g., subunit B of either cholera toxin or *E. coli* heat-labile toxin. Several possibilities are can be used for achieving fusion. First, the polypeptide of the invention can be fused to the N-, or preferably, to the C-terminal end of the polypeptide having adjuvant.

activity. Second, a polypeptide fragment of the invention can be fused within the amino acid sequence of the polypeptide having adjuvant activity.

As stated above, the polynucleotides of the invention encode *Chlamydia* polypeptides in precursor or mature form. They can also encode hybrid precursors containing 5 heterologous signal peptides, which can mature into polypeptides of the invention. By "heterologous signal peptide" is meant a signal peptide that is not found in the naturally-occurring precursor of a polypeptide of the invention.

A polynucleotide of the invention, having a homologous coding sequence, hybridizes, preferably under stringent conditions, to a polynucleotide having a sequence as 10 shown in SEQ ID NOS:1 to 2. Hybridization procedures are, e.g., described in Ausubel *et al.*, (Ref 41), Silhavy *et al.* (Ref 43); Davis *et al.* (ref 44). Important parameters that can be considered for optimizing hybridization conditions are reflected in a formula that allows calculation of a critical value, the melting temperature above which two complementary DNA strands separate from each other Ref 45). This formula is as follows:  $T_m = 81.5 + 0.5 \times (\% G+C) + 1.6 \log (\text{positive ion concentration}) - 0.6 \times (\% \text{ formamide})$ . Under appropriate stringency conditions, hybridization temperature (Th) is approximately 20 to 40°C, 20 to 25°C, or, preferably 30 to 40°C below the calculated  $T_m$ . Those skilled in the art will understand that optimal temperature and salt conditions can be readily determined empirically in preliminary experiments using conventional procedures.

For example, stringent conditions can be achieved, both for pre-hybridizing and hybridizing incubations, (i) within 4-16 hours at 42°C, in 6 x SSC containing 50% formamide or (ii) within 4-16 hours at 65°C in an aqueous 6 x SSC solution (1 M NaCl, 0.1 M sodium citrate (pH 7.0)).

For polynucleotides containing 30 to 600 nucleotides, the above formula is used and 25 then is corrected by subtracting (600/polynucleotide size in base pairs). Stringency conditions are defined by a Th that is 5 to 10°C below  $T_m$ .

Hybridization conditions with oligonucleotides shorter than 20-30 bases do not exactly follow the rules set forth above. In such cases, the formula for calculating the  $T_m$  is as follows:  $T_m = 4 \times (G+C) + 2 (A+T)$ . For example, an 18 nucleotide fragment of 30 50% G+C would have an approximate  $T_m$  of 54°C.

A polynucleotide molecule of the invention, containing RNA, DNA, or modifications or combinations thereof, can have various applications. For example, a DNA molecule can be used (i) in a process for producing the encoded polypeptide in a recombinant host system, (ii) in the construction of vaccine vectors such as poxviruses, which are further used in methods and compositions for preventing and/or treating *Chlamydia* infection, (iii) as a vaccine agent (as well as an RNA molecule), in a naked form or formulated with a delivery vehicle and, (iv) in the construction of attenuated *Chlamydia* strains that can over-express a polynucleotide of the invention or express it in a non-toxic, mutated form.

According to a second aspect of the invention, there is therefore provided (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required for expression, in particular under the control of an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a prokaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention, which involves culturing a prokaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, under conditions that allow expression of the DNA molecule of the invention and, recovering the encoded polypeptide or polypeptide derivative from the cell culture.

A recombinant expression system can be selected from prokaryotic and eucaryotic hosts. Eucaryotic hosts include yeast cells (e.g., *Saccharomyces cerevisiae* or *Pichia pastoris*), mammalian cells (e.g., COS1, NIH3T3, or JEG3 cells), arthropods cells (e.g., *Spodoptera frugiperda* (SF9) cells), and plant cells. Preferably, a prokaryotic host such as *E. coli* is used. Bacterial and eucaryotic cells are available from a number of different sources to those skilled in the art, e.g., the American Type Culture Collection (ATCC; Rockville, Maryland).

The choice of the expression system depends on the features desired for the expressed polypeptide. For example, it may be useful to produce a polypeptide of the invention in a particular lipidated form or any other form.

The choice of the expression cassette will depend on the host system selected as well as the features desired for the expressed polypeptide. Typically, an expression cassette includes a promoter that is functional in the selected host system and can be constitutive or

inducible; a ribosome binding site; a start codon (ATG) if necessary, a region encoding a signal peptide, *e.g.*, a lipidation signal peptide; a DNA molecule of the invention; a stop codon; and optionally a 3' terminal region (translation and/or transcription terminator). The signal peptide encoding region is adjacent to the polynucleotide of the invention and placed in proper reading frame. The signal peptide-encoding region can be homologous or heterologous to the DNA molecule encoding the mature polypeptide and can be specific to the secretion apparatus of the host used for expression. The open reading frame constituted by the DNA molecule of the invention, solely or together with the signal peptide, is placed under the control of the promoter so that transcription and translation occur in the host system. Promoters, signal peptide encoding regions are widely known and available to those skilled in the art and includes, for example, the promoter of *Salmonella typhimurium* (and derivatives) that is inducible by arabinose (promoter araB) and is functional in Gram-negative bacteria such as *E. coli* (as described in U.S. Patent No. 5,028,530 and in Cagnon *et al.*, (Ref 46); the promoter of the gene of bacteriophage T7 encoding RNA polymerase, that is functional in a number of *E. coli* strains expressing T7 polymerase (described in U.S. Patent No. 4,952,496); OspA lipidation signal peptide ; and RlpB lipidation signal peptide (Ref 47).

The expression cassette is typically part of an expression vector, which is selected for its ability to replicate in the chosen expression system. Expression vectors (*e.g.*, plasmids or viral vectors) can be chosen from those described in Pouwels *et al.* (Cloning Vectors: A Laboratory Manual 1985, Supp. 1987). They can be purchased from various commercial sources.

Methods for transforming/transfected host cells with expression vectors will depend on the host system selected as described in Ausubel *et al.*, (Ref 41).

Upon expression, a recombinant polypeptide of the invention (or a polypeptide derivative) is produced and remains in the intracellular compartment, is secreted/excreted in the extracellular medium or in the periplasmic space, or is embedded in the cellular membrane. The polypeptide can then be recovered in a substantially purified form from the cell extract or from the supernatant after centrifugation of the recombinant cell culture. Typically, the recombinant polypeptide can be purified by antibody-based affinity purification or by any other method that can be readily adapted by a person skilled in the art, such as by genetic fusion to a small affinity binding domain. Antibody-based affinity purification

methods are also available for purifying a polypeptide of the invention extracted from a *Chlamydia* strain. Antibodies useful for purifying by immunoaffinity the polypeptides of the invention can be obtained as described below.

5 A polynucleotide of the invention can also be useful in the vaccine field, *e.g.*, for achieving DNA vaccination. There are two major possibilities, either using a viral or bacterial host as gene delivery vehicle (live vaccine vector) or administering the gene in a free form, *e.g.*, inserted into a plasmid. Therapeutic or prophylactic efficacy of a polynucleotide of the invention can be evaluated as described below.

10 Accordingly, in a third aspect of the invention, there is provided (i) a vaccine vector such as a poxvirus, containing a DNA molecule of the invention, placed under the control of elements required for expression; (ii) a composition of matter containing a vaccine vector of the invention, together with a diluent or carrier; particularly, (iii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a vaccine vector of the invention; (iv) a method for inducing an immune response against *Chlamydia* in a mammal (*e.g.*, a human; alternatively, the method can be used in veterinary applications for treating or preventing *Chlamydia* infection of animals, *e.g.*, cats or birds), which involves administering to the mammal an immunologically effective amount of a vaccine vector of the invention to elicit an immune response, *e.g.*, a protective or therapeutic immune response to *Chlamydia*; and, particularly, (v) a method for preventing and/or treating a *Chlamydia* (*e.g.*, *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, *C. pecorum*) infection, which involves administering a prophylactic or therapeutic amount of a vaccine vector of the invention to an individual in need. Additionally, the third aspect of the invention encompasses the use of a vaccine vector of the invention in the preparation of a medicament for preventing and/or treating *Chlamydia* infection.

25 A vaccine vector of the invention can express one or several polypeptides or derivatives of the invention, as well as at least one additional *Chlamydia* antigen, fragment, homolog, mutant, or derivative thereof. In addition, it can express a cytokine, such as interleukin-2 (IL-2) or interleukin-12 (IL-12), that enhances the immune response (adjuvant effect). Thus, a vaccine vector can include an additional DNA sequence encoding, *e.g.*, a chlamydial antigen, or a cytokine, placed under the control of elements required for expression in a mammalian cell.

Alternatively, a composition of the invention can include several vaccine vectors, each of them being capable of expressing a polypeptide or derivative of the invention. A composition can also contain a vaccine vector capable of expressing an additional *Chlamydia* antigen, or a subunit, fragment, homolog, mutant, or derivative thereof; or a cytokine such as 5 IL-2 or IL-12.

In vaccination methods for treating or preventing infection in a mammal, a vaccine vector of the invention can be administered by any conventional route in use in the vaccine field, particularly, to a mucosal (e.g., ocular, intranasal, oral, gastric, pulmonary, intestinal, rectal, vaginal, or urinary tract) surface or via the parenteral (e.g., subcutaneous, intradermal, 10 intramuscular, intravenous, or intraperitoneal) route. Preferred routes depend upon the choice of the vaccine vector. The administration can be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters understood by skilled artisans such as the vaccine vector itself, the route of administration or the condition of the mammal to be vaccinated (weight, age and the like).

Live vaccine vectors available in the art include viral vectors such as adenoviruses and poxviruses as well as bacterial vectors, e.g., *Shigella*, *Salmonella*, *Vibrio cholerae*, *Lactobacillus*, Bacille bilié de Calmette-Guérin (BCG), and *Streptococcus*.

An example of an adenovirus vector, as well as a method for constructing an adenovirus vector capable of expressing a DNA molecule of the invention, are described in U.S. Patent No. 4,920,209. Poxvirus vectors that can be used include, e.g., vaccinia and canary pox virus, described in U.S. Patent No. 4,722,848 and U.S. Patent No. 5,364,773, respectively (also see, e.g., Tartaglia *et al.*, *Virology* (1992) 188:217) for a description of a vaccinia virus vector; and Taylor *et al.*, *Vaccine* (1995) 13:539 for a reference of a canary pox). Poxvirus vectors capable of expressing a polynucleotide of the invention can be 25 obtained by homologous recombination as described in Kiency *et al.*, *Nature* (1984) 312:163 so that the polynucleotide of the invention is inserted in the viral genome under appropriate conditions for expression in mammalian cells. Generally, the dose of vaccine viral vector, for therapeutic or prophylactic use, can be of from about  $1 \times 10^4$  to about  $1 \times 10^{11}$ , advantageously from about  $1 \times 10^7$  to about  $1 \times 10^{10}$ , preferably of from about  $1 \times 10^7$  to about  $1 \times 10^9$  plaque-forming units per kilogram. Preferably, viral vectors are administered parenterally; for 30 example, in 3 doses, 4 weeks apart. Those skilled in the art recognize that it is preferable to

avoid adding a chemical adjuvant to a composition containing a viral vector of the invention and thereby minimizing the immune response to the viral vector itself.

Non-toxicogenic *Vibrio cholerae* mutant strains that are useful as a live oral vaccine are described in Mekalanos *et al.*, *Nature* (1983) 306:551 and U.S. Patent No. 4,882,278 (strain in which a substantial amount of the coding sequence of each of the two *ctxA* alleles has been deleted so that no functional *cholerae* toxin is produced); WO 92/11354 (strain in which the *irgA* locus is inactivated by mutation; this mutation can be combined in a single strain with *ctxA* mutations); and WO 94/1533 (deletion mutant lacking functional *ctxA* and *ctxRS1* DNA sequences). These strains can be genetically engineered to express heterologous antigens, as described in WO 94/19482. An effective vaccine dose of a *Vibrio cholerae* strain capable of expressing a polypeptide or polypeptide derivative encoded by a DNA molecule of the invention can contain, *e.g.*, about  $1 \times 10^5$  to about  $1 \times 10^9$ , preferably about  $1 \times 10^6$  to about  $1 \times 10^8$  viable bacteria in an appropriate volume for the selected route of administration. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

Attenuated *Salmonella typhimurium* strains, genetically engineered for recombinant expression of heterologous antigens, or not, and their use as oral vaccines, are described in Nakayama *et al.*, *Bio/Technology* (1988) 6:693, and WO 92/11361. Preferred routes of administration include all mucosal routes; most preferably, these vectors are administered intranasally or orally.

Others bacterial strains useful as vaccine vectors are described in High *et al.*, EMBO (1992) 11:1991 and Sizemore *et al.*, *Science* (1995) 270:299 (*Shigella flexneri*); Medaglini *et al.*, *Proc. Natl. Acad. Sci. USA* (1995) 92:6868 (*Streptococcus gordonii*); and Flynn J.L., *Cell. Mol. Biol.* (1994) 40 (suppl. I):31, WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796, and WO 92/21376 (Bacille Calmette Guerin).

In bacterial vectors, polynucleotide of the invention can be inserted into the bacterial genome or can remain in a free state, carried on a plasmid.

An adjuvant can also be added to a composition containing a vaccine bacterial vector. A number of adjuvants are known to those skilled in the art. Preferred adjuvants can be selected from the list provided below.

According to a fourth aspect of the invention, there is also provided (i) a composition of matter containing a polynucleotide of the invention, together with a diluent or carrier; (ii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a polynucleotide of the invention; (iii) a method for inducing an immune response against *Chlamydia*, in a mammal, by administering to the mammal, an immunogenically effective amount of a polynucleotide of the invention to elicit an immune response, *e.g.*, a protective immune response to *Chlamydia*; and particularly, (iv) a method for preventing and/or treating a *Chlamydia* (*e.g.*, *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, or *C. pecorum*) infection, by administering a prophylactic or therapeutic amount of a polynucleotide of the invention to an individual in need. Additionally, the fourth aspect of the invention encompasses the use of a polynucleotide of the invention in the preparation of a medicament for preventing and/or treating *Chlamydia* infection. The fourth aspect of the invention preferably includes the use of a DNA molecule placed under conditions for expression in a mammalian cell, *e.g.*, in a plasmid that is unable to replicate in mammalian cells and to substantially integrate in a mammalian genome.

Polynucleotides (DNA or RNA) of the invention can also be administered as such to a mammal for vaccine, *e.g.*, therapeutic or prophylactic, purpose. When a DNA molecule of the invention is used, it can be in the form of a plasmid that is unable to replicate in a mammalian cell and unable to integrate in the mammalian genome. Typically, a DNA molecule is placed under the control of a promoter suitable for expression in a mammalian cell. The promoter can function ubiquitously or tissue-specifically. Examples of non-tissue specific promoters include the early Cytomegalovirus (CMV) promoter (described in U.S. Patent No. 4,168,062) and the Rous Sarcoma Virus promoter (described in Norton & Coffin, Molec. Cell Biol. (1985) 5:281). The desmin promoter (Li *et al.*, Gene (1989) 78:243, Li & Paulin, J. Biol. Chem. (1991) 266:6562 and Li & Paulin, J. Biol. Chem. (1993) 268:10403) is tissue-specific and drives expression in muscle cells. More generally, useful vectors are described, *i.a.*, WO 94/21797 and Hartikka *et al.*, Human Gene Therapy (1996) 7:1205.

For DNA/RNA vaccination, the polynucleotide of the invention can encode a precursor or a mature form. When it encodes a precursor form, the precursor form can be homologous or heterologous. In the latter case, a eucaryotic leader sequence can be used, such as the leader sequence of the tissue-type plasminogen factor (tPA).

A composition of the invention can contain one or several polynucleotides of the invention. It can also contain at least one additional polynucleotide encoding another *Chlamydia* antigen such as urease subunit A, B, or both; or a fragment, derivative, mutant, or analog thereof. A polynucleotide encoding a cytokine, such as interleukin-2 (IL-2) or interleukin-12 (IL-12), can also be added to the composition so that the immune response is enhanced. These additional polynucleotides are placed under appropriate control for expression. Advantageously, DNA molecules of the invention and/or additional DNA molecules to be included in the same composition, can be carried in the same plasmid.

Standard techniques of molecular biology for preparing and purifying polynucleotides can be used in the preparation of polynucleotide therapeutics of the invention. For use as a vaccine, a polynucleotide of the invention can be formulated according to various methods.

First, a polynucleotide can be used in a naked form, free of any delivery vehicles, such as anionic liposomes, cationic lipids, microparticles, e.g., gold microparticles, precipitating agents, e.g., calcium phosphate, or any other transfection-facilitating agent. In this case, the polynucleotide can be simply diluted in a physiologically acceptable solution, such as sterile saline or sterile buffered saline, with or without a carrier. When present, the carrier preferably is isotonic, hypotonic, or weakly hypertonic, and has a relatively low ionic strength, such as provided by a sucrose solution, e.g., a solution containing 20% sucrose.

Alternatively, a polynucleotide can be associated with agents that assist in cellular uptake. It can be, i.a., (i) complemented with a chemical agent that modifies the cellular permeability, such as bupivacaine (see, e.g., WO 94/16737); (ii) encapsulated into liposomes, or (iii) associated with cationic lipids or silica, gold, or tungsten microparticles.

Anionic and neutral liposomes are well-known in the art (see, e.g., *Liposomes: A Practical Approach*, RPC New Ed, IRL press (1990), for a detailed description of methods for making liposomes) and are useful for delivering a large range of products, including polynucleotides.

Cationic lipids are also known in the art and are commonly used for gene delivery. Such lipids include Lipofectin™ also known as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethylammonio)propane), DDAB (dimethyldioctadecylammonium bromide), DOGS

(dioctadecylamidoglycyl spermine) and cholesterol derivatives such as DC-Chol (3 beta-(N-(N',N'-dimethyl aminomethane)-carbamoyl) cholesterol). A description of these cationic lipids can be found in EP 187,702, WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5,527,928. Cationic lipids for gene delivery are 5 preferably used in association with a neutral lipid such as DOPE (dioleyl phosphatidylethanolamine), as, for example, described in WO 90/11092.

Other transfection-facilitating compounds can be added to a formulation containing cationic liposomes. A number of them are described in, *e.g.*, WO 93/18759, WO 93/19768, WO 94/25608, and WO 95/2397. They include, *i.a.*, spermine derivatives useful for 10 facilitating the transport of DNA through the nuclear membrane (see, for example, WO 93/18759) and membrane-permeabilizing compounds such as GALA, Gramicidine S, and cationic bile salts (see, for example, WO 93/19768).

Gold or tungsten microparticles can also be used for gene delivery, as described in WO 91/359, WO 93/17706, and Tang *et al.* (Nature (1992) 356:152). In this case, the microparticle-coated polynucleotides can be injected *via* intradermal or intraepidermal routes using a needleless injection device ("gene gun"), such as those described in U.S. Patent No. 4,945,050, U.S. Patent No. 5,015,580, and WO 94/24263.

The amount of DNA to be used in a vaccine recipient depends, *e.g.*, on the strength of the promoter used in the DNA construct, the immunogenicity of the expressed gene product, the condition of the mammal intended for administration (*e.g.*, the weight, age, and 20 general health of the mammal), the mode of administration, and the type of formulation. In general, a therapeutically or prophylactically effective dose from about 1  $\mu$ g to about 1 mg, preferably, from about 10  $\mu$ g to about 800  $\mu$ g and, more preferably, from about 25  $\mu$ g to about 250  $\mu$ g, can be administered to human adults. The administration can be achieved in a single 25 dose or repeated at intervals.

The route of administration can be any conventional route used in the vaccine field. As general guidance, a polynucleotide of the invention can be administered *via* a mucosal surface, *e.g.*, an ocular, intranasal, pulmonary, oral, intestinal, rectal, vaginal, and urinary tract surface; or *via* a parenteral route, *e.g.*, by an intravenous, subcutaneous, intraperitoneal, 30 intradermal, intraepidermal, or intramuscular route. The choice of the administration route will depend on, *e.g.*, the formulation that is selected. A polynucleotide formulated in

association with bupivacaine is advantageously administered into muscles. When a neutral or anionic liposome or a cationic lipid, such as DOTMA or DC-Chol, is used, the formulation can be advantageously injected *via* intravenous, intranasal (aerosolization), intramuscular, intradermal, and subcutaneous routes. A polynucleotide in a naked form can advantageously 5 be administered *via* the intramuscular, intradermal, or sub-cutaneous routes.

Although not absolutely required, such a composition can also contain an adjuvant. If so, a systemic adjuvant that does not require concomitant administration in order to exhibit an adjuvant effect is preferable such as, *e.g.*, QS21, which is described in U.S. Patent No. 5,057,546.

10 The sequence information provided in the present application enables the design of specific nucleotide probes and primers that can be useful in diagnosis. Accordingly, in a fifth aspect of the invention, there is provided a nucleotide probe or primer having a sequence found in or derived by degeneracy of the genetic code from a sequence shown in SEQ ID NO:1 to 2.

15 The term "probe" as used in the present application refers to DNA (preferably single stranded) or RNA molecules (or modifications or combinations thereof) that hybridize under the stringent conditions, as defined above, to nucleic acid molecules having sequences homologous to those shown in SEQ ID NOs:1 and 2, or to a complementary or anti-sense sequence. Generally, probes are significantly shorter than full length sequences shown in SEQ: 20 ID NOs:1 and 2; for example, they can contain from about 5 to about 100, preferably from about 10 to about 80 nucleotides. In particular, probes have sequences that are at least 75%, preferably at least 85%, more preferably 95% homologous to a portion of a sequence as shown in SEQ ID NOs:1 and 2 or that are complementary to such sequences. Probes can contain modified bases such as inosine, methyl-5-deoxycytidine, deoxyuridine, dimethylamino- 25 5-deoxyuridine, or diamino-2, 6-purine. Sugar or phosphate residues can also be modified or substituted. For example, a deoxyribose residue can be replaced by a polyamide (Nielsen *et al.*, Science (1991) 254:1497) and phosphate residues can be replaced by ester groups such as diphosphate, alkyl, arylphosphonate and phosphorothioate esters. In addition, the 2'-hydroxyl group on ribonucleotides can be modified by including, *e.g.*, alkyl groups.

30 Probes of the invention can be used in diagnostic tests, as capture or detection probes. Such capture probes can be conventionally immobilized on a solid support, directly or

indirectly, by covalent means or by passive adsorption. A detection probe can be labelled by a detection marker selected from radioactive isotopes; enzymes such as peroxidase, alkaline phosphatase, and enzymes able to hydrolyze a chromogenic, fluorogenic, or luminescent substrate; compounds that are chromogenic, fluorogenic, or luminescent; nucleotide base 5 analogs; and biotin.

Probes of the invention can be used in any conventional hybridization technique, such as dot blot (Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), Southern blot (Southern, J. Mol. Biol. (1975) 98:503), northern blot (identical to Southern blot to the exception that RNA 10 is used as a target), or the sandwich technique (Dunn *et al.*, Cell (1977) 12:23). The latter technique involves the use of a specific capture probe and/or a specific detection probe with nucleotide sequences that at least partially differ from each other.

A primer is usually a probe of about 10 to about 40 nucleotides that is used to initiate enzymatic polymerization of DNA in an amplification process (*e.g.*, PCR), in an elongation process, or in a reverse transcription method. In a diagnostic method involving PCR, primers can be labelled.

Thus, the invention also encompasses (i) a reagent containing a probe of the invention for detecting and/or identifying the presence of *Chlamydia* in a biological material; (ii) a method for detecting and/or identifying the presence of *Chlamydia* in a biological material, in which (a) a sample is recovered or derived from the biological material, (b) DNA or RNA is extracted from the material and denatured, and (c) exposed to a probe of the invention, for example, a capture, detection probe or both, under stringent hybridization conditions, such that hybridization is detected; and (iii) a method for detecting and/or identifying the presence of *Chlamydia* in a biological material, in which (a) a sample is recovered or derived from the biological material, (b) DNA is extracted therefrom, (c) the extracted DNA is primed with at least one, and preferably two, primers of the invention and amplified by polymerase chain reaction, and (d) the amplified DNA fragment is produced.

As previously mentioned, polypeptides that can be produced upon expression of the newly identified open reading frames are useful vaccine agents.

Therefore, a sixth aspect of the invention features a substantially purified polypeptide or polypeptide derivative having an amino acid sequence encoded by a polynucleotide of the invention.

A "substantially purified polypeptide" is defined as a polypeptide that is separated from the environment in which it naturally occurs and/or that is free of the majority of the polypeptides that are present in the environment in which it was synthesized. For example, a substantially purified polypeptide is free from cytoplasmic polypeptides. Those skilled in the art will understand that the polypeptides of the invention can be purified from a natural source, i.e., a *Chlamydia* strain, or can be produced by recombinant means.

Homologous polypeptides or polypeptide derivatives encoded by polynucleotides of the invention can be screened for specific antigenicity by testing cross-reactivity with an antiserum raised against the polypeptide of reference having an amino acid sequence as shown in SEQ ID NOs:3 to 4. Briefly, a monospecific hyperimmune antiserum can be raised against a purified reference polypeptide as such or as a fusion polypeptide, for example, an expression product of MBP, GST, or His-tag systems or a synthetic peptide predicted to be antigenic. The homologous polypeptide or derivative screened for specific antigenicity can be produced as such or as a fusion polypeptide. In this latter case, and if the antiserum is also raised against a fusion polypeptide, two different fusion systems are employed. Specific antigenicity can be determined according to a number of methods, including Western blot (Towbin, et al., Proc. Natl. Acad. Sci. USA (1979) 76:4350), dot blot, and ELISA, as described below.

In a Western blot assay, the product to be screened, either as a purified preparation or a total *E. coli* extract, is submitted to SDS-PAGE electrophoresis as described by Laemmli (Nature (1970) 227:680). After transfer to a nitrocellulose membrane, the material is further incubated with the monospecific hyperimmune antiserum diluted in the range of dilutions from about 1:5 to about 1:5000, preferably from about 1:100 to about 1:500. Specific antigenicity is shown once a band corresponding to the product exhibits reactivity at any of the dilutions in the above range.

In an ELISA assay, the product to be screened is preferably used as the coating antigen. A purified preparation is preferred, although a whole cell extract can also be used. Briefly, about 100  $\mu$ l of a preparation at about 10  $\mu$ g protein/ml are distributed into wells of a 96-well polycarbonate ELISA plate. The plate is incubated for 2 hours at 37°C then overnight

at 4°C. The plate is washed with phosphate buffer saline (PBS) containing 0.05% Tween 20 (PBS/Tween buffer). The wells are saturated with 250  $\mu$ l PBS containing 1% bovine serum albumin (BSA) to prevent non-specific antibody binding. After 1 hour incubation at 37°C, the plate is washed with PBS/Tween buffer. The antiserum is serially diluted in PBS/Tween buffer containing 0.5% BSA. 100  $\mu$ l of dilutions are added per well. The plate is incubated for 90 minutes at 37°C, washed and evaluated according to standard procedures. For example, a goat anti-rabbit peroxidase conjugate is added to the wells when specific antibodies were raised in rabbits. Incubation is carried out for 90 minutes at 37°C and the plate is washed. The reaction is developed with the appropriate substrate and the reaction is measured by colorimetry (absorbance measured spectrophotometrically). Under the above experimental conditions, a positive reaction is shown by O.D. values greater than a non immune control serum.

In a dot blot assay, a purified product is preferred, although a whole cell extract can also be used. Briefly, a solution of the product at about 100  $\mu$ g/ml is serially two-fold diluted in 50 mM Tris-HCl (pH 7.5). 100  $\mu$ l of each dilution are applied to a nitrocellulose membrane 0.45  $\mu$ m set in a 96-well dot blot apparatus (Biorad). The buffer is removed by applying vacuum to the system. Wells are washed by addition of 50 mM Tris-HCl (pH 7.5) and the membrane is air-dried. The membrane is saturated in blocking buffer (50 mM Tris-HCl (pH 7.5) 0.15 M NaCl, 10 g/L skim milk) and incubated with an antiserum dilution from about 1:50 to about 1:5000, preferably about 1:500. The reaction is revealed according to standard procedures. For example, a goat anti-rabbit peroxidase conjugate is added to the wells when rabbit antibodies are used. Incubation is carried out 90 minutes at 37°C and the blot is washed. The reaction is developed with the appropriate substrate and stopped. The reaction is measured visually by the appearance of a colored spot, e.g., by colorimetry. Under the above experimental conditions, a positive reaction is shown once a colored spot is associated with a dilution of at least about 1:5, preferably of at least about 1:500.

Therapeutic or prophylactic efficacy of a polypeptide or derivative of the invention can be evaluated as described below.

According to a seventh aspect of the invention, there is provided (i) a composition of matter containing a polypeptide of the invention together with a diluent or carrier; in particular, (ii) a pharmaceutical composition containing a therapeutically or prophylactically

effective amount of a polypeptide of the invention; (iii) a method for inducing an immune response against *Chlamydia* in a mammal, by administering to the mammal an immunogenically effective amount of a polypeptide of the invention to elicit an immune response, *e.g.*, a protective immune response to *Chlamydia*; and particularly, (iv) a method for preventing and/or treating a *Chlamydia* (*e.g.*, *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, or *C. pecorum*) infection, by administering a prophylactic or therapeutic amount of a polypeptide of the invention to an individual in need. Additionally, the seventh aspect of the invention encompasses the use of a polypeptide of the invention in the preparation of a medicament for preventing and/or treating *Chlamydia* infection.

The immunogenic compositions of the invention can be administered by any conventional route in use in the vaccine field, in particular to a mucosal (*e.g.*, ocular, intranasal, pulmonary, oral, gastric, intestinal, rectal, vaginal, or urinary tract) surface or via the parenteral (*e.g.*, subcutaneous, intradermal, intramuscular, intravenous, or intraperitoneal) route. The choice of the administration route depends upon a number of parameters, such as the adjuvant associated with the polypeptide. For example, if a mucosal adjuvant is used, the intranasal or oral route will be preferred and if a lipid formulation or an aluminum compound is used, the parenteral route will be preferred. In the latter case, the subcutaneous or intramuscular route is most preferred. The choice can also depend upon the nature of the vaccine agent. For example, a polypeptide of the invention fused to CTB or LTB will be best administered to a mucosal surface.

A composition of the invention can contain one or several polypeptides or derivatives of the invention. It can also contain at least one additional *Chlamydia* antigen, or a subunit, fragment, homolog, mutant, or derivative thereof.

For use in a composition of the invention, a polypeptide or derivative thereof can be formulated into or with liposomes, preferably neutral or anionic liposomes, microspheres, ISCOMS, or virus-like-particles (VLPs) to facilitate delivery and/or enhance the immune response. These compounds are readily available to one skilled in the art; for example, see *Liposomes: A Practical Approach (supra)*.

Adjuvants other than liposomes and the like can also be used and are known in the art. An appropriate selection can conventionally be made by those skilled in the art, for example, from the list provided below.

Administration can be achieved in a single dose or repeated as necessary at intervals as can be determined by one skilled in the art. For example, a priming dose can be followed by three booster doses at weekly or monthly intervals. An appropriate dose depends on various parameters including the recipient (*e.g.*, adult or infant), the particular vaccine 5 antigen, the route and frequency of administration, the presence/absence or type of adjuvant, and the desired effect (*e.g.*, protection and/or treatment), as can be determined by one skilled in the art. In general, a vaccine antigen of the invention can be administered by a mucosal route in an amount from about 10  $\mu$ g to about 500 mg, preferably from about 1 mg to about 200 mg. For the parenteral route of administration, the dose usually should not exceed about 10 1 mg, preferably about 100  $\mu$ g.

When used as vaccine agents, polynucleotides and polypeptides of the invention can be used sequentially as part of a multistep immunization process. For example, a mammal can be initially primed with a vaccine vector of the invention such as a pox virus, *e.g.*, *via* the parenteral route, and then boosted twice with the polypeptide encoded by the vaccine vector, *e.g.*, *via* the mucosal route. In another example, liposomes associated with a polypeptide or derivative of the invention can also be used for priming, with boosting being carried out mucosally using a soluble polypeptide or derivative of the invention in combination with a mucosal adjuvant (*e.g.*, LT).

A polypeptide derivative of the invention is also useful as a diagnostic reagent for detecting the presence of anti-*Chlamydia* antibodies, *e.g.*, in a blood sample. Such polypeptides are about 5 to about 80, preferably about 10 to about 50 amino acids in length and can be labeled or unlabeled, depending upon the diagnostic method. Diagnostic methods involving such a reagent are described below.

Upon expression of a DNA molecule of the invention, a polypeptide or polypeptide 25 derivative is produced and can be purified using known laboratory techniques. For example, the polypeptide or polypeptide derivative can be produced as a fusion protein containing a fused tail that facilitates purification. The fusion product can be used to immunize a small mammal, *e.g.*, a mouse or a rabbit, in order to raise antibodies against the polypeptide or polypeptide derivative (monospecific antibodies). The eighth aspect of the invention thus 30 provides a monospecific antibody that binds to a polypeptide or polypeptide derivative of the invention.

By "monospecific antibody" is meant an antibody that is capable of reacting with a unique naturally-occurring *Chlamydia* polypeptide. An antibody of the invention can be polyclonal or monoclonal. Monospecific antibodies can be recombinant, *e.g.*, chimeric (*e.g.*, constituted by a variable region of murine origin associated with a human constant region), 5 humanized (a human immunoglobulin constant backbone together with hypervariable region of animal, *e.g.*, murine, origin), and/or single chain. Both polyclonal and monospecific antibodies can also be in the form of immunoglobulin fragments, *e.g.*, F(ab)2 or Fab fragments. The antibodies of the invention can be of any isotype, *e.g.*, IgG or IgA, and polyclonal antibodies can be of a single isotype or can contain a mixture of isotypes.

10 The antibodies of the invention, which are raised to a polypeptide or polypeptide derivative of the invention, can be produced and identified using standard immunological assays, *e.g.*, Western blot analysis, dot blot assay, or ELISA (see, *e.g.*, Coligan *et al.*, Current Protocols in Immunology (1994) John Wiley & Sons, Inc., New York, NY). The antibodies can be used in diagnostic methods to detect the presence of a *Chlamydia* antigen in a sample, such as a biological sample. The antibodies can also be used in affinity chromatography methods for purifying a polypeptide or polypeptide derivative of the invention. As is discussed further below, such antibodies can be used in prophylactic and therapeutic passive immunization methods.

20 Accordingly, a ninth aspect of the invention provides (i) a reagent for detecting the presence of *Chlamydia* in a biological sample that contains an antibody, a polypeptide, or a polypeptide derivative of the invention; and (ii) a diagnostic method for detecting the presence of *Chlamydia* in a biological sample, by contacting the biological sample with an antibody, a polypeptide, or a polypeptide derivative of the invention, such that an immune complex is formed, and by detecting such complex to indicate the presence of *Chlamydia* in the sample or 25 the organism from which the sample is derived.

Those skilled in the art will understand that the immune complex is formed between a component of the sample and the antibody, polypeptide, or polypeptide derivative, whichever is used, and that any unbound material can be removed prior to detecting the complex. As can be easily understood, a polypeptide reagent is useful for detecting the 30 presence of anti-*Chlamydia* antibodies in a sample, *e.g.*, a blood sample, while an antibody of

the invention can be used for screening a sample, such as a gastric extract or biopsy, for the presence of *Chlamydia* polypeptides.

For use in diagnostic applications, the reagent (*i.e.*, the antibody, polypeptide, or polypeptide derivative of the invention) can be in a free state or immobilized on a solid support, such as a tube, a bead, or any other conventional support used in the field. Immobilization can be achieved using direct or indirect means. Direct means include passive adsorption (non-covalent binding) or covalent binding between the support and the reagent. By "indirect means" is meant that an anti-reagent compound that interacts with a reagent is first attached to the solid support. For example, if a polypeptide reagent is used, an antibody that binds to it can serve as an anti-reagent, provided that it binds to an epitope that is not involved in the recognition of antibodies in biological samples. Indirect means can also employ a ligand-receptor system, for example, a molecule such as a vitamin can be grafted onto the polypeptide reagent and the corresponding receptor can be immobilized on the solid phase. This is illustrated by the biotin-streptavidin system. Alternatively, indirect means can be used, *e.g.*, by adding to the reagent a peptide tail, chemically or by genetic engineering, and immobilizing the grafted or fused product by passive adsorption or covalent linkage of the peptide tail.

According to a tenth aspect of the invention, there is provided a process for purifying, from a biological sample, a polypeptide or polypeptide derivative of the invention, which involves carrying out antibody-based affinity chromatography with the biological sample, wherein the antibody is a monospecific antibody of the invention.

For use in a purification process of the invention, the antibody can be polyclonal or monospecific, and preferably is of the IgG type. Purified IgGs can be prepared from an antiserum using standard methods (see, *e.g.*, Coligan *et al.*, *supra*). Conventional chromatography supports, as well as standard methods for grafting antibodies, are disclosed in, *e.g.*, Antibodies: A Laboratory Manual, D. Lane, E. Harlow, Eds. (1988).

Briefly, a biological sample, such as an *C. pneumoniae* extract, preferably in a buffer solution, is applied to a chromatography material, preferably equilibrated with the buffer used to dilute the biological sample so that the polypeptide or polypeptide derivative of the invention (*i.e.*, the antigen) is allowed to adsorb onto the material. The chromatography material, such as a gel or a resin coupled to an antibody of the invention, can be in batch form

or in a column. The unbound components are washed off and the antigen is then eluted with an appropriate elution buffer, such as a glycine buffer or a buffer containing a chaotropic agent, *e.g.*, guanidine HCl, or high salt concentration (*e.g.*, 3 M MgCl<sub>2</sub>). Eluted fractions are recovered and the presence of the antigen is detected, *e.g.*, by measuring the absorbance at 5 280 nm.

An antibody of the invention can be screened for therapeutic efficacy as described as follows. According to an eleventh aspect of the invention, there is provided (i) a composition of matter containing a monospecific antibody of the invention, together with a diluent or carrier, (ii) a pharmaceutical composition containing a therapeutically or prophylactically effective amount of a monospecific antibody of the invention, and (iii) a 10 method for treating or preventing a *Chlamydia* (*e.g.*, *C. trachomatis*, *C. psittaci*, *C. pneumoniae* or *C. pecorum*) infection, by administering a therapeutic or prophylactic amount of a monospecific antibody of the invention to an individual in need. Additionally, the eleventh aspect of the invention encompasses the use of a monospecific antibody of the invention in the preparation of a medicament for treating or preventing *Chlamydia* infection.

To this end, the monospecific antibody can be polyclonal or monoclonal, preferably of the IgA isotype (predominantly). In passive immunization, the antibody can be administered to a mucosal surface of a mammal, *e.g.*, the gastric mucosa, *e.g.*, orally or intragastrically, advantageously, in the presence of a bicarbonate buffer. Alternatively, systemic administration, not requiring a bicarbonate buffer, can be carried out. A monospecific antibody of the invention can be administered as a single active component or as a mixture with at least one monospecific antibody specific for a different *Chlamydia* polypeptide. The amount of antibody and the particular regimen used can be readily determined by one skilled in the art. For example, daily administration of about 100 to 1,000 25 mg of antibodies over one week, or three doses per day of about 100 to 1,000 mg of antibodies over two or three days, can be an effective regimens for most purposes.

Therapeutic or prophylactic efficacy can be evaluated using standard methods in the art, *e.g.*, by measuring induction of a mucosal immune response or induction of protective and/or therapeutic immunity, using, *e.g.*, the *C. pneumoniae* mouse model. Those skilled in 30 the art will recognize that the *C. pneumoniae* strain of the model can be replaced with another *Chlamydia* strain. For example, the efficacy of DNA molecules and polypeptides from *C.*

*pneumoniae* is preferably evaluated in a mouse model using an *C. pneumoniae* strain. Protection can be determined by comparing the degree of *Chlamydia* infection to that of a control group. Protection is shown when infection is reduced by comparison to the control group. Such an evaluation can be made for polynucleotides, vaccine vectors, polypeptides and derivatives thereof, as well as antibodies of the invention.

5 Adjuvants useful in any of the vaccine compositions described above are as follows.

10 Adjuvants for parenteral administration include aluminum compounds, such as aluminum hydroxide, aluminum phosphate, and aluminum hydroxy phosphate. The antigen can be precipitated with, or adsorbed onto, the aluminum compound according to standard protocols. Other adjuvants, such as RIBI (ImmunoChem, Hamilton, MT), can be used in parenteral administration.

15 Adjuvants for mucosal administration include bacterial toxins, *e.g.*, the cholera toxin (CT), the *E. coli* heat-labile toxin (LT), the *Clostridium difficile* toxin A and the *pertussis* toxin (PT), or combinations, subunits, toxoids, or mutants thereof. For example, a purified preparation of native cholera toxin subunit B (CTB) can be of use. Fragments, homologs, derivatives, and fusions to any of these toxins are also suitable, provided that they retain adjuvant activity. Preferably, a mutant having reduced toxicity is used. Suitable mutants are described, *e.g.*, in WO 95/17211 (Arg-7-Lys CT mutant), WO 96/6627 (Arg-192-Gly LT mutant), and WO 95/34323 (Arg-9-Lys and Glu-129-Gly PT mutant). Additional LT mutants that can be used in the methods and compositions of the invention include, *e.g.*, Ser-63-Lys, Ala-69-Gly, Glu-110-Asp, and Glu-112-Asp mutants. Other adjuvants, such as a bacterial monophosphoryl lipid A (MPLA) of, *e.g.*, *E. coli*, *Salmonella minnesota*, *Salmonella typhimurium*, or *Shigella flexneri*; saponins, or polylactide glycolide (PLGA) microspheres, can also be used in mucosal administration.

25 Adjuvants useful for both mucosal and parenteral administrations include polyphosphazene (WO 95/2415), DC-chol (3 b-(N-(N',N'-dimethyl aminomethane)-carbamoyl) cholesterol; U.S. Patent No. 5,283,185 and WO 96/14831) and QS-21 (WO 88/9336).

30 Any pharmaceutical composition of the invention, containing a polynucleotide, a polypeptide, a polypeptide derivative, or an antibody of the invention, can be manufactured in a conventional manner. In particular, it can be formulated with a pharmaceutically acceptable

diluent or carrier, *e.g.*, water or a saline solution such as phosphate buffer saline. In general, a diluent or carrier can be selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers or diluents, as well as pharmaceutical necessities for their use in pharmaceutical formulations, are described in 5 *Remington's Pharmaceutical Sciences*, a standard reference text in this field and in the USP/NF.

The invention also includes methods in which *Chlamydia* infection are treated by oral administration of a *Chlamydia* polypeptide of the invention and a mucosal adjuvant, in combination with an antibiotic, an antacid, sucralfate, or a combination thereof. Examples of 10 such compounds that can be administered with the vaccine antigen and the adjuvant are antibiotics, including, *e.g.*, macrolides, tetracyclines, and derivatives thereof (specific examples of antibiotics that can be used include azithromycin or doxycyclin or immunomodulators such as cytokines or steroids. In addition, compounds containing more than one of the above-listed components coupled together, can be used. The invention also includes compositions for carrying out these methods, *i.e.*, compositions containing a *Chlamydia* antigen (or antigens) of the invention, an adjuvant, and one or more of the above-listed compounds, in a pharmaceutically acceptable carrier or diluent.

Amounts of the above-listed compounds used in the methods and compositions of the invention can readily be determined by one skilled in the art. In addition, one skilled in the art can readily design treatment/immunization schedules. For example, the non-vaccine components can be administered on days 1-14, and the vaccine antigen + adjuvant can be administered on days 7, 14, 21, and 28.

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Figure 1

tagcttggaa tagttccctc caattgtat ttctgaagaa gtataggggg aatgtcga 60  
gagatagtct tgttttaag gaggaggaa aaacggttt atg agc aga aaa gac 115  
Met Ser Arg Lys Asp  
Arg Lys Asp  
1 5  
aat gag gtt tcc tta gct cgt tca att ttt aat ata tta tcc gga act 163  
Asn Glu Val Ser Leu Ala Arg Ser Ile Phe Asn Ile Leu Ser Gly Thr  
Asn Glu Val Ser Leu Ala Arg Ser Ile Phe Asn Ile Leu Ser Gly Thr  
10 15 20  
ttc tgg agt cgt att aca ggg ata ttt cga gaa att gca atg gca acc 211  
Phe Cys Ser Arg Ile Thr Gly Ile Phe Arg Glu Ile Ala Met Ala Thr  
Phe Cys Ser Arg Ile Thr Gly Ile Phe Arg Glu Ile Ala Met Ala Thr  
25 30 35  
tat ttt gga gct gat cca att gta gct gtc ttc tgg tta ggt ttc cgt 259  
Tyr Phe Gly Ala Asp Pro Ile Val Ala Ala Phe Trp Leu Gly Phe Arg  
Tyr Phe Gly Ala Asp Pro Ile Val Ala Ala Phe Trp Leu Gly Phe Arg  
40 45 50  
act gtt ttt ttc tta aga aaa att tta gga ggg ctc att cta gaa caa 307  
Thr Val Phe Phe Leu Arg Lys Ile Leu Gly Gly Leu Ile Leu Glu Gln  
Thr Val Phe Phe Leu Arg Lys Ile Leu Gly Gly Leu Ile Leu Glu Gln  
55 60 65  
gcc ttc atc cct cat ttt gaa ttt ctc cgt gct caa agt ctc gat cgt 355  
Ala Phe Ile Pro His Phe Glu Phe Leu Arg Ala Gln Ser Leu Asp Arg  
Ala Phe Ile Pro His Phe Glu Phe Leu Arg Ala Gln Ser Leu Asp Arg  
70 75 80 85  
gct gct ttt ttc cga cgc ttt tct aga ttg att aaa ggc agc act 403  
Ala Ala Phe Phe Arg Arg Phe Ser Arg Leu Ile Lys Gly Ser Thr  
Ala Ala Phe Phe Arg Arg Phe Ser Arg Leu Ile Lys Gly Ser Thr  
90 95 100  
att ata ttc act ctg ctt att gaa gca gta ttg tgg gta ttc ttc aat 451  
Ile Ile Phe Thr Leu Leu Ile Glu Ala Val Leu Trp Val Phe Phe Asn  
Ile Ile Phe Thr Leu Leu Ile Glu Ala Val Leu Trp Val Phe Phe Asn  
105 110 115  
aac gtt gaa gag ggg act tac gat atg att ctc ctt act atg ata ctc 499  
Asn Val Glu Glu Gly Thr Tyr Asp Met Ile Leu Leu Thr Met Ile Leu  
Asn Val Glu Glu Gly Thr Tyr Asp Met Ile Leu Leu Thr Met Ile Leu  
120 125 130  
ttt ccc tgt ggc att ttc tta atg atg tac aat gta aac ggc gct ttg 547  
Leu Pro Cys Gly Ile Phe Leu Met Met Tyr Asn Val Asn Gly Ala Leu  
Leu Pro Cys Gly Ile Phe Leu Met Met Tyr Asn Val Asn Gly Ala Leu  
135 140 145  
ctt cac tgt gga aat aag ttt ttc ggg gtg gga tta gct ccc gta gtt 595  
Leu His Cys Gly Asn Lys Phe Phe Gly Val Gly Leu Ala Pro Val Val  
Leu His Cys Gly Asn Lys Phe Phe Gly Val Gly Leu Ala Pro Val Val  
150 155 160 165  
gta aat atc att tgg att ttc ttt gtt ata gcg gct cgt cat tca gat 643  
Val Asn Ile Ile Trp Ile Phe Phe Val Ile Ala Ala Arg His Ser Asp  
Val Asn Ile Ile Trp Ile Phe Phe Val Ile Ala Ala Arg His Ser Asp  
170 175 180

cct aga gag cgt att atc ggt tta tcc gtg gct cta gtt atc ggg ttt	691
Pro Arg Glu Arg Ile Ile Gly Leu Ser Val Ala Leu Val Ile Gly Phe	
Pro Arg Glu Arg Ile Ile Gly Leu Ser Val Ala Leu Val Ile Gly Phe	
185 190 195	
ttc ttc gaa tgg tta atc acg gtt cct gga gta tgg aaa ttt cta tta	739
Phe Phe Glu Trp Leu Ile Thr Val Pro Gly Val Trp Lys Phe Leu Leu	
Phe Phe Glu Trp Leu Ile Thr Val Pro Gly Val Trp Lys Phe Leu Leu	
200 205 210	
gaa ggc aag agc cca ctt caa gaa cac gat agt gtt cga gct tta tta	787
Glu Ala Lys Ser Pro Pro Gln His Asp Ser Val Arg Ala Leu Leu	
Glu Ala Lys Ser Pro Pro Gln His Asp Ser Val Arg Ala Leu Leu	
215 220 225	
gtc ccc tta tct ttg ggt att tta act tca agc atc ttc cag ctg aac	835
Ala Pro Leu Ser Leu Gly Ile Leu Thr Ser Ser Ile Phe Gln Leu Asn	
Ala Pro Leu Ser Leu Gly Ile Leu Thr Ser Ser Ile Phe Gln Leu Asn	
230 235 240 245	
ctt ctt tct gat atc tgc ttg gct cgc tat gta cat gaa ata ggc cct	883
Leu Leu Ser Asp Ile Cys Leu Ala Arg Tyr Val His Glu Ile Gly Pro	
Leu Leu Ser Asp Ile Cys Leu Ala Arg Tyr Val His Glu Ile Gly Pro	
250 255 260	
cta tat ctt atg tac tcc tta aag att tat cag ctc ccc ata cat ctc	931
Leu Tyr Leu Met Tyr Ser Leu Lys Ile Tyr Gln Leu Pro Ile His Leu	
Leu Tyr Leu Met Tyr Ser Leu Lys Ile Tyr Gln Leu Pro Ile His Leu	
265 270 275	
ttt ggc ttt ggt gtg ttt acc gtt ctc ctc cca gca att tct cgt tgt	979
Phe Gly Phe Gly Val Phe Thr Val Leu Leu Pro Ala Ile Ser Arg Cys	
Phe Gly Phe Gly Val Phe Thr Val Leu Leu Pro Ala Ile Ser Arg Cys	
280 285 290	
gta cag cga gaa gat cat gag agg gga ttg aaa ctt atg aag ttc gtt	1027
Val Gln Arg Glu Asp His Glu Arg Gly Leu Lys Leu Met Lys Phe Val	
Val Gln Arg Glu Asp His Glu Arg Gly Leu Lys Leu Met Lys Phe Val	
295 300 305	
ctc acc cta acc atg tcc gta atg atc att atg acg gca ggg cta ttg	1075
Leu Thr Leu Thr Met Ser Val Met Ile Ile Met Thr Ala Gly Leu Leu	
Leu Thr Leu Thr Met Ser Val Met Ile Ile Met Thr Ala Gly Leu Leu	
310 315 320 325	
ctc tta gct tta cct gga gtc cgt gtc ctt tat gaa cac gga ctt ttc	1123
Leu Leu Ala Leu Pro Gly Val Arg Val Leu Tyr Glu His Gly Leu Phe	
Leu Leu Ala Leu Pro Gly Val Arg Val Leu Tyr Glu His Gly Leu Phe	
330 335 340	
cct cag agt gct tac gct att gtt cgt gta ttg cga ggt tat ggt	1171
Pro Gln Ser Ala Val Tyr Ala Ile Val Arg Val Leu Arg Gly Tyr Gly	
Pro Gln Ser Ala Val Tyr Ala Ile Val Arg Val Leu Arg Gly Tyr Gly	
345 350 355	
gcc agt att atc cct atg gcc ttg gct cct tta gtc tct gtt ctt ttt	1219
Ala Ser Ile Ile Pro Met Ala Leu Ala Pro Leu Val Ser Val Leu Phe	
Ala Ser Ile Ile Pro Met Ala Leu Ala Pro Leu Val Ser Val Leu Phe	
360 365 370	
tat gca cag cgg cag tat gct gtt ccg ctc ttt ataggatggatggatcgc	1267
Tyr Ala Gln Arg Gln Tyr Ala Val Pro Leu Phe Ile Gly Ile Gly Thr	
Tyr Ala Gln Arg Gln Tyr Ala Val Pro Leu Phe Ile Gly Ile Gly Thr	

375

380

385

gct ttg gcc aat att gtt tta agc ttg gtt cta ggt cgt tgg gtt tta Ala Leu Ala Asn Ile Val Leu Ser Leu Val Leu Gly Arg Trp Val Leu Ala Leu Ala Asn Ile Val Leu Ser Leu Val Leu Gly Arg Trp Val Leu 390 395 400 405	1315
aaa gac gtc tcg ggc att tcc tat gct aca tcc ata act gct tgg gtg Lys Asp Val Ser Gly Ile Ser Tyr Ala Thr Ser Ile Thr Ala Trp Val Lys Asp Val Ser Gly Ile Ser Tyr Ala Thr Ser Ile Thr Ala Trp Val 410 415 420	1363
cag tta tat ttc ctc tgg tat tat tct tcg aaa aga ctc cct atg tac Gln Leu Tyr Phe Leu Trp Tyr Ser Ser Lys Arg Leu Pro Met Tyr Gln Leu Tyr Phe Leu Trp Tyr Ser Ser Lys Arg Leu Pro Met Tyr 425 430 435	1411
tct aag tta ctt tgg gag agc atc cgg cgt tcc ata aaa gtt atg gga Ser Lys Leu Leu Trp Glu Ser Ile Arg Arg Ser Ile Lys Val Met Gly Ser Lys Leu Leu Trp Glu Ser Ile Arg Arg Ser Ile Lys Val Met Gly 440 445 450	1459
acc act atg ctt gct tgt atg att act cta ggc tta aat atc ctt acg Thr Thr Met Leu Ala Cys Met Ile Thr Leu Gly Leu Asn Ile Leu Thr Thr Thr Met Leu Ala Cys Met Ile Thr Leu Gly Leu Asn Ile Leu Thr 455 460 465	1507
caa act aca tat gta att ttc tta aac ccc ctc aca cca ctt gct tgg Gln Thr Thr Tyr Val Ile Phe Leu Asn Pro Leu Thr Pro Leu Ala Trp Gln Thr Thr Tyr Val Ile Phe Leu Asn Pro Leu Thr Pro Leu Ala Trp 470 475 480 485	1555
ccc tta tcc tcc ata acg gct caa gca att gct ttt tta tct gag agc Pro Leu Ser Ser Ile Thr Ala Gln Ala Ile Ala Phe Leu Ser Glu Ser Pro Leu Ser Ser Ile Thr Ala Gln Ala Ile Ala Phe Leu Ser Glu Ser 490 495 500	1603
tgc att ttc ttg gct ttt ttg ttt ggt ttt gca aaa ctg ctt cga gta Cys Ile Phe Leu Ala Phe Leu Phe Gly Phe Ala Lys Leu Leu Arg Val Cys Ile Phe Leu Ala Phe Leu Phe Gly Phe Ala Lys Leu Leu Arg Val 505 510 515	1651
gaa gat ctt att aat ttg gct tct ttt gaa tac tgg cgt ggg caa cgg Glu Asp Leu Ile Asn Leu Ala Ser Phe Glu Tyr Trp Arg Gly Gln Arg Glu Asp Leu Ile Asn Leu Ala Ser Phe Glu Tyr Trp Arg Gly Gln Arg 520 525 530	1699
ggt ctt ttg caa aga caa cac gtg atg caa gac act caa aat Gly Leu Leu Gln Arg Gln His Val Met Gln Asp Thr Gln Asn Gly Leu Leu Gln 535 540 545	1741
taatcatgtt tgtttcttgt agctcagtcg ctttcttta gctttaagtt ttgatagcct 1801	
gcttggcttt ctgtttctac acttaatatt gatactaagg atactatgaa aaaacaggtt 1861	
tatcaatggt tagcgagtgt ggttctttta gcgctgaca 1900	

Figure 2

## Restriction enzyme analysis of CPN100557

TaqI

AluI	AluI	MunI	Hpy188IX			Eco57I	
CviJI	CviJI	Tsp509I	MnII			MboII	EarI
TAGCTTGAAATAGCTTCCTCCAATTGTGATTCTGAAGAAGTATAGGGGGAAATGTCGAA							

1 1-----+-----+-----+-----+-----+-----+-----+ 60

ATCGAAGCTTATCGAAGGAGGTAAACACTAAAGACTTCTCATATGCCCGTTAGAGCTT

MnII

DraI			MseI				
MseI			BseRI				
MnII			TaaI				
MboII							MnII
GAGATAGCTTGTTAAAGGAGGAGGGAAACGGTTAATGAGCAGAAAAGACAATGA							

61 1-----+-----+-----+-----+-----+-----+-----+ 120

CTCTATCAGAACAAAATTCCTCCTCCCTTGCCAAATTACTCGTCTTCTGTTACT

AluI Hpy178III

CviJI			MspI				
Bpu10I			BsaWI				
DdeI	Tsp509I	MseI	BspEI				SfcI
GGTTCCCTAGCTCGTTCAATTAAATATATTATCCGAACTTCTGTAGTCGTATTAC							

121 1-----+-----+-----+-----+-----+-----+-----+ 180

CCAAAGGAATCGAGGAAGTTAAAAATTATATAATAGGCCTTGAAAGACATCAGGATAATG,

MunI

Tsp509I			DpnI				
			BbvI				Fnu4HI
			Sau3AI				AluI
AGGGATATTCGAGAAATTGCAATGGCAACCTATTTGGAGCTGATCCAATTGTAGCTGC							

181 1-----+-----+-----+-----+-----+-----+-----+ 240

TCCCTATAAAGCTTTAACGTTACCGTTGGATAAAACCTCGACTAGGTTAACATCGACG

Hpy178III

MseI	MnII	BanII	BfaI				
TaaI	AflII	ApoI	Bsp1286I				
RsaI	SmlI	Tsp509I	FokI				
			CviJI				
			XbaI				
TTTCTGGTTAGGTTCCGTACTGTTTTCTTAAGAAAAATTAGGAGGGCTCATTCT							

241 1-----+-----+-----+-----+-----+-----+-----+ 300

AAAGACCAATCCAAAGGCATGACAAAAAAAGAATTCTTTAAAATCCTCCGAGTAAGA

Fnu4HI

Bsp1286I

PvuI

DpnI

BsmAI

Sau3AI

MnlI Hpy178III

ApoI BsiHKAI

CviJI Tsp509I Bsp1286I

TauI

AcII

Hpy178III Fnu4HI

BfaI TseI

HgaI MmeI

Hpy188IX XbaI MseI

BbvI

MnlI

AcII

EarI

MaeII

MboII

BsmFI

HinfI

Tfil

RleAI MboII

TATTGAAGCAGTATTGGGGTATTCTCAATAACGTTGAAGAGGGGACTTACGATATGAT

ATAACTCGTCATAACACCCATAAGAAGTTATTGCAACTTCTCCCTGAATGCTATACTA

RsaI

BsrGI

MseI TatI

Sth132I

TspRI

HaeII TaaI

HhaI Bcefi

BscGI

AluI

CviJI

Sth132I

BfaI

DpnI

CviJI BstYI

Fnu4HI Sau3AI

TauI Hpy188IX

AcII AlwI

AGAACAGCCTTCATCCCTCATTTGAATTCTCCGTGCTCAAAGTCTCGATCGTGCAGGC

TCTTGTTCGGAAGTAGGGAGTAAAGCTAAAGAGGCACGAGTTCAGAGCTAGCACGCCG

301 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 360

GTTTTTTTCCGACGCTTTCTAGATTGATTAAAGGCAGCACTATTATATTCACTCTGCT

CAAAAAAAAGGCTGCAGAAAGATCTAACTAATTCCGTGATAATATAAGTGAGACGA

361 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 420

TCTCCTTACTATGATACTCTGCCCTGGCATTCTTAATGATGTACAATGTAAACGG

AGAGGAATGATACTATGAGAACGGGACACGGTAAAGAATTACTACATCTTACATTGCC

481 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 540

CGCTTTGTTCACTGTGGAAATAAGTTTCCGGGTGGGATTAGCTCCGTAGTTGTAAA

GCGAACGAAGTGACACCTTATTCAAAAGCCCCACCTAACGAGGGCATCAACATTT

541 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 600

TATCATTGGATTCTTGTATAGCGGCTCGTCATTAGATCCTAGAGAGCGTATTAT

601 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 660

ATAGTAAACCTAAAAGAAAACAATATCGCCGAGCAGTAAGTCTAGGATCTCTCGCATAATA

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      BfaI           ScrFI
      Sth132I        CjeI           EcoRII
      CviJI          NspV           NlaIV
      BsaJI          MboII          TaqI           MseI           TaaI
      BstDSI          |           |           |           |
      |           |           |           |           |
      CGGTTTATCCGTGGCTCTAGTTATCGGGTTTCTTCGAATGGTTAACGGTTCCTGG
661 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 720
GCCAAATAGGGACGGACATCAATAGGCCAAAAGAAGGCTAACCAATTAGTCGGAAAGGACG

```

	AluI						
MseI	CviJI	AceIII	CvIJI	BseRI	TaaI		
901	CTTAAAGATTATCAGCTCCCCATACATCTTTGGCTTGGTGTGTTACCGTTCTCCT						960
	GAATTTCTAAATAGTCGAGGGGTATGTAGAGAAACCGAACACAAATGGCAAGAGGA						

MboII  
NlaIII  
HpaI 78101

RcaI | | |  
 RsaI DpnI | | |  
 MnII BsrGI | MnII | | |  
 Tsp509I | TATI | Sau3AI | | |  
 CCCAGCAATTCTCGTTGTACAGCGAGAAGATCATGAGAGGGATTGAAACTTATGAA  
 961 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1020  
 GGGTCGTTAACAGAGCAACACATGTCGCTCTTAGTACTCTCCCTAACCTTGAATACTT  
  
 DpnI  
 BclI |  
 HphI NlaIII Sau3AI | | |  
 CviJI DdeI | | |  
 GTTCGTTCTCACCTAACCATGTCGGTAATGATCATTATGACAGCAGGGCTATTGCTCTT  
 1021 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1080  
 CAAGCAAGAGTGGGATTGGTACAGGCATTACTAGTAATACTGTCGTCGGATAACGAGAA  
  
 BsaXI  
 Hin4I  
 HinfI |  
 ScrFI | | |  
 EcoRII | | |  
 AluI | | |  
 CviJI | | |  
 PstI | | |  
 BpmI |  
 Hpy188IX |  
 DdeI | | |  
 BseMII |  
 MwoI |  
 AccI |  
 MnII | | |  
 AGCTTTACCTGGAGTCGGTGTCTTATGAACACGGACTTTCCCTCAGAGTGTGCTCTA  
 1081 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1140  
 TCGAAATGGACCTCAGGACAGGAAATACTTGTGCCTGAAAAGGGAGTCTCACGACAGAT  
  
 BsaJI  
 StyI  
 CviJI |  
 BsrI |  
 NlaIV |  
 MnII |  
 BanI |  
 HaeI | | NlaIV  
 HaeIII | CviJI |  
 CGCTATTGTCGTGTATTGCGAGGTTATGGTGCAGTATTATCCCTATGGCCTGGCTCC  
 1141 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1200  
 GCGATAACAAGCACATAACGCTCCAATACCACGGTCATAATAGGGATAACCGAACCGAGG  
  
 Fnu4HI  
 TauI  
 AcI |  
 BsmAI |  
 CviRI | | |  
 MspAII | | |  
 BsrBI |  
 AcI |  
 HinfI |  
 Tfil |  
 TTTAGTCTCTGTTCTTTATGCACAGCGGAGTATGCTGTCGCTCTTATAGGAAT  
 1201 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1260  
 AAATCAGAGACAAGAAAAATACGTGTCGCCGTACAGACAAGGCAGAAATATCCTTA  
  
 CviJI | | |  
 AluI | | |  
 HaeI | | |  
 HaeIII | | |  
 CviJI | | |  
 HindIII | | |  
 BsrBI | | |  
 BfaI | | |  
 RsaI | | |  
 EaeI | | |  
 MscI | | |  
 BceI | | |  
 SspI | | |  
 MseI | | |  
 DrdII | | |  
 BsaHI |  
 MaeII |  
 Sth132I |  
 DraI | | |  
 MseI | | |  
 CGGTACGGCTTGGCCAATATTGTTTAAGCTGGTCTAGTCGTTGGGTTAAAAGA

1261 -----+-----+-----+-----+-----+ 1320  
 GCCATGCCGAAACCGGTTATAACAAAATCGAACCAAGATCCAGCAACCCAAAATTTCT

FokI  
 BsmAI  
 BsmBI  
 AvaI |  
 AatII | | |  
 | | | TagII  
 | | | Tth111II |  
 | | | | |  
 CGTCTCGGGCATTCTATGCTACATCCATAACTGCTGGGTGCAGTTATATTCCTCTG  
 1321 -----+-----+-----+-----+-----+ 1380  
 GCAGAGCCCGTAAAGGATACGATGTAGGTATTGACGAACCCACGTCAATATAAAGGAGAC

PleI  
 MnII NspV | FokI  
 BsgI | TaqI | HinFI RsaI MaeIII |  
 | | | TatI | DdeI | | |  
 | | | | | | |  
 GTATTATTCTTCGAAAAGACTCCCTATGTACTCTAAGTTACTTTGGGAGAGCATCCGGCG  
 1381 -----+-----+-----+-----+-----+ 1440  
 CATAATAAGAAGCTTTCTGAGGGATACATGAGATTCAATGAAACCCCTCTCGTAGGCCGC

Tth111II  
 DrdII  
 NlaIV |  
 Tth111II | |  
 XcmI | | | Cac8I |  
 | | | | |  
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 1441 -----+-----+-----+-----+-----+ 1500  
 AAGGTATTTCAATACCCCTGGTGATACGAAGGAACATACTAATGAGATCCGAATTATA

Bce83I  
 CjePI  
 CviJI  
 HaeIII  
 Sau96I |  
 Tsp509I | | | |  
 NdeI | MseI | Tth111II | MnII |  
 | | | | |  
 CCTTACGCAAACATACATATGTAATTTCTTAAACCCCTCAGACCACTTGGTGGCCCTT  
 1501 -----+-----+-----+-----+-----+ 1560  
 GGAATGCGTTGATGTATACTAAAAGAATTGGGGAGTGTGGTGAACGAACCGGAA

DdeI  
 Hpy188IX  
 Tth111II  
 MunI CjePI | CviRI  
 Tsp509I BbvI | Fnu4HI |  
 SmlI AceIII | AluI |  
 CviJI BcefI | CviJI | CviJI  
 MnII BseMII | TseI | Tth111II |  
 | | | | | | |  
 ATCCCTCCATAACGGCTCAAGCAATTGCTTTTATCTGAGAGCTGCATTTCTGGCTTT  
 1561 -----+-----+-----+-----+-----+ 1620  
 TAGGAGGTATTGCCGAGTCGTTAACGAAAAATAGACTCTGACGTAAAAGAACCGAAA

DpnI | MboII  
BglII | Tsp509I  
BstYI | MseI ||  
CviRI | TaqI | Sau3AI | VspI || CviJI

TTTGGTTGGTTTGC~~AAA~~ACTGCTTCGACTAGAAGATCTTATTAA~~TTGGCTT~~TTTG  
1621 -----+-----+-----+-----+-----+ 1680  
AAACAAACCAAAACGTTTGACGAAGCTCATCTCTAGAATAATTAAACCGAAGAAA~~ACT~~

BsaAI  
PmlI  
MaeII ||  
AflIII ||  
BsrI | SimI | SfaNI | | | Tsp509I  
Sth132I | BscGI | CviRI | BsbI | CviRI | Tth111II  
ATACTGGCGTGGGCAACGGGGCTTTGCAAAGACAACACGTGATGCAAGACACTAAAA  
1681 -----+-----+-----+-----+-----+ 1740  
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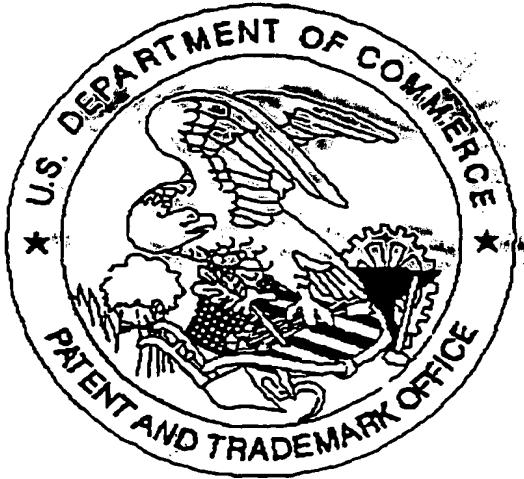
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MseI | BbsI  
DdeI | Cac8I  
AluI | MboII  
CviJI | CviJI  
VspI | NlaIII | CviJI | BseMII  
TTAACATGTTGTTCTTGAGCTCAGTCGCTTCTTTAGCTTAAGTTTGATAGCC  
1741 -----+-----+-----+-----+-----+ 1800  
AATTAGTACAAACAAAGAACATCGAGTCAGCGAAAGAAAATCGAAATTCAAAACTATCGG

SspI | DdeI  
MseI | BciVI |  
TGCTTGGTCTTCTGTTCTACACTTAATATTGATACTAAGGATACTATGAAAAAACAGGT  
1801 -----+-----+-----+-----+-----+ 1860  
ACGAACCAGAACAAAGATGTGAATTATAACTATGATTCCCTATGATACTTTTGTC~~CA~~

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HaeII  
HhaI ||  
DrdII | Eco47III ||  
ATATCAATGGTTAGCGAGTGTGGTTCTTTAGCGCTGACA  
1861 -----+-----+-----+-----+ 1900  
TATAGTTACCAATCGCTCACACCAAGAAAATCGCGACTGT

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